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In vitro activity of *Quercus brantii* extracts against biofilm-producing *Pseudomonas aeruginosa*

Nasim Kashef[1,*], Zahra Sadat Seyyedi[1], Ahmad Reza Gohari[2]

[1] *Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran*

[2] *Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Biofilm formation by *Pseudomonas aeruginosa* is a serious concern in treatment of diseases and medical industries. Natural products that originate in plants can influence microbial biofilm formation. The effect of ethyl acetate, methanol and water-methanol extracts of *Quercus brantii* on biofilm formation and biofilm disruption of *P. aeruginosa* were investigated in this study. The effect of *Q. brantii* extracts on biofilm formation ability of *P. aeruginosa* (ATCC 27853) and clinical isolates was tested using crystal violet (CV) staining assay. Minimum biofilm eradication concentration (MBEC) of the extracts against pre-formed biofilms alone and in combination with N-acetylcysteine (NAC) was also investigated. Ethyl acetate extract of *Q. brantii* was the most effective extract and inhibited *P. aeruginosa* biofilm formation over 70%. It was followed by water-methanol (52-66% inhibition) and methanol extracts (44-57% inhibition). Water-methanol extract of *Q. brantii* was more effective in eradication of *P. aeruginosa* pre-formed biofilms. The MBEC of *Q. brantii* extracts in combination with NAC was decreased in comparison to MBEC of *Q. brantii* extracts alone. This study demonstrated that *Q. brantii* extracts had a good inhibitory effect on biofilm formation ability of *P. aeruginosa* and could eradicate preformed-biofilms in combination with NAC.

Keywords: Bacteria; Biofilm formation; Biofilm eradication; Plant extract; *Pseudomonas aeruginosa*.

Introduction

The emergence of antibiotic resistant pathogens has become a global threat to public healthcare, resulting in increased costs and decreased efficiency of treatments. Biofilm formation is one of the main sources of resistance in pathogens causing hospital infections. Biofilm is a group of microorganisms that grows on living and non-living surfaces and surrounded by an extracellular matrix to provide an important barrier against the antibiotic therapies and immune system defense (1).

Due to the reduced activity of antibiotics to eliminate the biofilm, it is one of the main problems in industry and medicine. *Pseudomonas aeruginosa* is

* **Corresponding author:** kashefn@khayam.ut.ac.ir

one of the main bacteria in biofilm formation. It is a human opportunistic pathogen, which is considered as one of the most important causes of nosocomial infections. *Pseudomonas aeruginosa* often causes infections in burns and wounds and is a major problem for immunocompromised individuals (2). Chronic lung infection in patients with cystic fibrosis was the first biofilm infection described in humans and is now the most well-studied biofilm infection caused by *P. aeruginosa* (3). Given the high prevalence of *P. aeruginosa* in nosocomial infections and the failure of antibiotics in complete elimination of this pathogen, it is necessary to find alternative or complementary therapies (2).

Almost all nations and cultures have written some texts about extracting essential oils from plant for medicinal use. Plants by producing a wide range of chemicals, in addition to having antimicrobial activity against planktonic cells, are able to weaken the biofilm through specific mechanisms (4).

Quercus, a genus of the Fagaceae plant family, contains 500 species worldwide. *Quercus brantii* L. is predominant in western regions of Iran. The fruit of oak tree, known as acorn, comprises vitamins, nutrients, and carbohydrates in relatively large amounts. Acorn also contains considerable amounts of phenolic, tannin, catechin, epicathechin, and gallocatechin components (5-8). There are reports representing different biological activities of some species of the genus *Quercus* (9, 10) including antibacterial (11, 12), and antiviral ones (13, 14) by different species of this genus.

There have been a few reports on the anti-biofilm activity of *Quercus* extracts. Therefore, this research was aimed to prepare ethyl acetate, methanol and water-methanol extracts of *Q. brantii* acorn and to evaluate activity of these plant materials on biofilm formation and biofilm disruption of *P. aeruginosa*.

Materials and methods

Isolates

Pseudomonas aeruginosa (ATCC 27853) and 15 clinical isolates were used in this study. Clinical isolates retrospectively collected from burn wound infections. The isolates were confirmed by University of Tehran Microorganisms Collection (UTMC) and received in skim milk culture. Then clinical strains were cultured onto nutrient agar plates and incubated at 37°C for 24h. Antibiotic susceptibility of isolates to Imipenem (IMP10), Ceftazidim (CAZ30), Pipracillin-tazobactam (PIP100), Gentamycin (GM10), Ciprofloxacin (CP5), and Amikacin (AN30) was examined by disk-diffusion method (15).

Preparation of plant material

The fresh fruits of *Q. brantii* were collected from Prov. Khuzestan, Iran. Identification was confirmed by the herbarium of the Faculty of Agriculture, Shahid Chamran University (Ahwaz). The fruits were initially air-dried at room temperature and ground to a fine powder in a blender. The pulverized materials (1 g) were extracted by maceration using 10 mL ethyl acetate (EtOAc) to obtain crude EtOAc extract. The plant materials (1 g) were also extracted with 10 mL methanol (MeOH) to obtain crude MeOH extract. Same process was followed to prepare water-methanol (methanol: water, 50:50 v/v) extract. Furthermore, extracts were dried and concentrated under reduced pressure using a rotary evaporator to yield solid/ semisolid residues. Stock solutions of crude extracts were prepared, filter-sterilized (0.2 µm) and stored at 4°C (16-18).

Minimum inhibitory concentration of the extracts

Minimum Inhibitory Concentration (MIC) of ethyl acetate, methanol and water-methanol extracts of *Quercus brantii* against *P. aeruginosa* (ATCC 27853) was determined by micro-dilution method (18).

Evaluation of biofilm formation

Assessment of biofilm formation was performed using a microtitre plate assay according to Perez et al. (19). Briefly, 1 ml of bacterial suspension (1 McFarland standard) was added to 2 ml of Trypticase Soy Broth (TSB) and incubated at 37°C for 24h. After incubation, the culture was vortexed and thereafter diluted 1:50 in fresh TSB, and 200 µl of this solution was incubated in 96-well flat-bottomed plates at 37°C for 24h. Media with suspended bacteria was then removed; the plates were carefully washed two times

with sterile phosphate buffer saline (pH 7.4). 200 µl of 95% ethanol was added to each well and air-dried before staining with 200 µl of 0.4% crystal violet solution for 15 min. After removing the dye solution and washing with water, the attached dye was solubilized with 10% acetic acid and the optical density at 492 nm was determined by a microtitre plate reader. TSB was used as a negative control (background absorbance). A biofilm-positive phenotype was defined as an optical density at 492 nm of ≥0.17 (19, 20).

Inhibition of biofilm formation

The effect of different concentration (½ MIC, MIC and 2 MIC) of ethyl acetate, methanol and water-methanol extracts of *Q. brantii* on biofilm formation ability was tested on polystyrene flat-bottomed microtitre plates. 1 ml of 1 McFarland standard suspension of the microorganisms was added to 2 ml of TSB and incubated for 24h at 37°C. After incubation, the stationary-phase culture was vortexed and there-after diluted 1:50 in fresh TSB, and 100 µl of this solution was added into the wells in the presence of 100 µl of each extract. Solvents were used as the negative controls. Following incubation, the biofilm biomass was assayed using the modified crystal violet staining assay, and the percentage inhibition was determined according to the following formula (21):

Percentage inhibition =

(OD Negative control – OD Experimental)/

(OD Negative control) ×100

Evaluation of Minimum Biofilm Eradication Concentration (MBEC)

To test the ability of the extracts to disruption of pre-formed biofilms, biofilms were formed in 96 well microtitre plates by aliquoting 100 µl of 1 McFarland suspension of *P. aeruginosa* diluted 1:50 in TSB, into the wells. After adding 100 µl fresh TSB, the plates were incubated for 24h at 37°C. Following incubation, supernatants were aspirated, and the wells were rinsed with sterile phosphate buffer saline (PBS, pH=7.4) to remove non-adherent bacteria. Then, 200 µl of ethyl acetate, methanol and water-methanol extracts of *Q. brantii* (that showed some degree of inhibiting cell attachment) was diluted in TSB and added to a final concentration of 25-400 mg/ml in the wells. Culture medium was used as the positive control. The plates were further incubated for 24h at 37°C. The biofilms were scraped carefully, and then vortexed for 30s to homogenize the samples. Treated and untreated samples were serially diluted, plated on the nutrient agar plates, and incubated for 24h at 37°C. The MBEC was the lowest concentration of the extract at which 99.99% or more of the initial inoculum was killed (21, 22).

Evaluation of Minimum Biofilm Eradication Concentration of Q. brantii extracts in combination with N-acetylcysteine (NAC)

N-acetylcystein (Hexal, Germany) was dissolved in water to make a 20 mg/ml stock solution. Biofilm formations were performed as described above. After removing the planktonic cells, 200 µl of NAC-extracts (final concentration of NAC: 10, 8, 5 mg/ml and final concentrations of extracts: 400-25 mg/ml) was added to each well and the plates were incubated for 24h at 37ºC. The biofilms were scraped carefully, and then vortexed for 30s to homogenize the samples. Treated and untreated samples were serially diluted, plated on the nutrient agar plates, and incubated for 24h at 37°C.

Data analysis

Statistical analysis was carried out using SPSS Version 19.0. The values were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to evaluate any significant differences between control and treated groups. $P < 0.05$ was considered statistically significant.

Results

Antibiotic susceptibility test

Among 15 clinical isolates, 14 strains were resistant to CAZ and CP, 13 strains to IPM and GM and 10 strains to PIP and AN. Seven strains showed complete resistance to 6 antibiotics. These strains were UTMC7, UTMC9, UTMC11, UTMC17, UTMC27, UTMC30, UTMC32.

Minimum inhibitory concentration of extracts

MIC values of ethyl acetate, methanol and water-methanol extracts of *Q. brantii* tested against *P. aeruginosa* (ATCC 27853) were 6.25, 3.125 and 3.125 mg/ml, respectively.

Evaluation of biofilm formation

As shown in Table 1, *P. aeruginosa* (ATCC 27853) and all clinical isolates had biofilm formation ability (OD≥0.17), but with different strength.

Inhibition of biofilm formation

Table 2 shows the percentage inhibition of biofilm formation by plant extracts. Ethyl acetate extract of *Q. brantii* was found to be more effective in inhibiting biofilm formation ability of *P. aeruginosa* than ethanol and water methanol extracts. All extracts in three concentrations (½ MIC, MIC and 2 MIC) showed some degree of inhibition. In most of cases, there were significant differences between treated and untreated groups ($p<0.05$).

Evaluation of MBEC

Pseudomonas aeruginosa (ATCC 27853) and five clinical isolates were evaluated in this test. The results showed that water-methanol extract of *Q. brantii* was more effective in eradication of *P. aeruginosa* pre-formed biofilms than ethanol and ethyl acetate extracts (Table 3). *Pseudomonas aeruginosa* (ATCC 27853) biofilm was the most resistant to be eradicated by the extracts.

Evaluation of MBEC of Q. brantii *extracts in combination with NAC*

Non-destructive concentrations of NAC on pre-formed biofilms of *P. aeruginosa* (ATCC 27853) and clinical isolates (except UTMC17) were 10 and 8 mg/ml, respectively. UTMC17 was susceptible to NAC (at concentrations ≥2.5 mg/ml). As shown in Table 4, the MBEC of *Q. brantii* extracts in combination with NAC was decreased in comparison to MBEC of *Q. brantii* extracts alone.

Discussion

In the present study, the effect of ethyl acetate, methanol and water- methanol extracts of *Q. brantii* on biofilm formation and biofilm disruption of *P. aeruginosa* were assessed. Our results indicated that all extracts at the concentrations used in this study could inhibit biofilm formation ability of the microorganisms with a high percentage. Microbial cell adhesion and organization within a polysaccharide matrix is an important step in biofilm formation (23). Therefore, the ability of plant extracts to inhibit the cell adhesion can result in preventing biofilm formation (24).

Table 1. Biofilm formation ability of *Pseudomonas aeruginosa* (ATCC 27853) and 15 clinical isolates according to crystal violet assay

P. aeruginosa	Mean ± SD (absorption at 492 nm)	*P. aeruginosa*	Mean ± SD (absorption at 492 nm)
ATCC 27853	2.86 ± 0.19	UTMC 13	2.30 ± 0.62
UTMC 1	2.35 ± 0.14	UTMC 14	0.84 ± 0.3
UTMC 6	2.58 ± 0.4	UTMC 17	0.92 ± 0.5
UTMC 7	1.14 ± 0.19	UTMC 21	1.38 ± 0.1
UTMC 8	1.52 ± 0.03	UTMC 27	2.01 ± 0.08
UTMC 9	2.24 ± 0.24	UTMC 30	2.58 ± 0.11
UTMC 11	0.52 ± 0.24	UTMC 32	2.23 ± 0.09
UTMC 12	1.41 ± 0.62	UTMC 41	1.31 ± 0.3

Table 2. Percentage inhibition of biofilm formation of *Pseudomonas aeruginosa* ATCC 27853 and clinical isolates by ethyl acetate, methanol and water-methanol extracts of *Quercus brantii*

P. aeruginosa	plant extracts	Inhibition of biofilm formation (%)*		
		1/2 MIC	MIC	2 MIC
ATCC 27853	ethyl acetate	76 ± 2.7	81 ± 1.2	82 ± 2.5
	methanol	24 ± 0.4	47 ± 1.5	60 ± 2
	water-methanol	-6±2.5	26 ± 0.7	5 ± 2
UTMC 6	ethyl acetate	77 ± 4.1	79 ± 0.9	80 ± 3.5
	methanol	-12±3	12 ± 2.6	48 ± 1.5
	water-methanol	58 ± 3	72 ± 1.6	77 ± 3.7
UTMC 7	ethyl acetate	73 ± 1.9	68 ± 2.6	70 ± 3.3
	methanol	5 ± 0.8	46 ± 2.1	13 ± 4.1
	water-methanol	-60±1.9	32 ± 2	18 ± 2.1
UTMC 9	ethyl acetate	89 ± 1.2	90 ± 0.2	91 ± 0.6
	methanol	78 ± 3.6	77 ± 3.5	68 ± 3
	water-methanol	42 ± 0.5	77 ± 0.8	71 ± 1.2
UTMC 11	ethyl acetate	87 ± 0.5	89 ± 0.4	90 ± 0.2
	methanol	36 ± 2.6	55 ± 3	81 ± 1.3
	water-methanol	73 ± 3.7	78 ± 3.1	80 ± 1.8
UTMC 13	ethyl acetate	56 ± 0.1	71 ± 2.8	75 ± 2.7
	methanol	19 ± 1.5	21 ± 1.6	30 ± 3.3
	water-methanol	72 ± 1.4	76 ± 2.6	81 ± 2.6
UTMC 17	ethyl acetate	60 ± 3.4	71 ± 1.8	69 ± 2.9
	methanol	66 ± 2	63 ± 2.6	51 ± 2
	water-methanol	69 ± 2.6	67 ± 2.1	65 ± 1.6
UTMC 27	ethyl acetate	71 ± 2	72 ± 2.8	74 ± 1.3
	methanol	58 ± 2.6	75 ± 0.5	64 ± 4
	water-methanol	75 ± 4	81 ± 3.2	85 ± 4.1
UTMC 30	ethyl acetate	90 ± 1	88 ± 2.3	88 ± 0.5
	methanol	78 ± 2.5	85 ± 1.5	84 ± 1.5
	water-methanol	84 ± 3.5	90 ± 1.7	88 ± 1
UTMC 32	ethyl acetate	84 ± 2.3	86 ± 1.7	85 ± 2
	methanol	51 ± 0.7	82 ± 1.5	78 ± 3.5
	water-methanol	70 ± 1.5	77 ± 4.5	39 ± 0.6
UTMC 41	ethyl acetate	15 ± 2	65 ± 1.6	34 ± 2.5
	methanol	32 ± 0.6	53 ± 0.8	51 ± 1
	water-methanol	45 ± 3.6	58 ± 2.8	75 ± 2.3

*Percentage inhibition is presented as means ± SD of three repetition of the crystal violet assay.

Table 3. Minimum biofilm eradication concentration of ethyl acetate, methanol and water-methanol extracts of *Quercus brantii* against *Pseudomonas aeruginosa* biofilms

P. aeruginosa	*Q. brantii* extracts		
	Ethyl acetate (mg/ml)	Methanol (mg/ml)	Water-methanol (mg/ml)
ATCC (27853)	0.4	0.4	0.4
UTMC 6	0.4	0.2	0.2
UTMC 9	0.1	0.4	0.1
UTMC 17	0.1	0.1	0.05
UTMC 27	0.2	0.4	0.2
UTMC 30	0.4	0.2	0.2

Table 4. Minimum biofilm eradication concentration of ethyl acetate, methanol and water-methanol extracts of *Quercus brantii* in combination with *N*-acetylcysteine against *Pseudomonas aeruginosa* biofilms

	***Q. brantii* extracts**								
	N-acetylcysteine (5 mg/ml)			*N*-acetylcysteine (8 mg/ml)			*N*-acetylcysteine (10 mg/ml)		
P. aeruginosa	Ethyl acetate	Methanol	Water-methanol	Ethyl acetate	Methanol	Water-methanol	Ethyl acetate	Methanol	Water-methanol
ATCC (27853)	0.4	0.4	0.4	0.2	0.2	0.2	0.025	0.05	0.1
UTMC 6	0.2	0.2	0.2	0.1	0.1	0.05	-	-	-
UTMC 9	0.1	0.1	0.1	0.05	0.05	0.1	-	-	-
UTMC 27	0.2	0.2	0.2	0.1	0.1	0.2	-	-	-
UTMC 30	0.2	0.1	0.2	0.2	0.05	0.1	-	-	-

(-) NAC at a concentration of 10 mg/ml was bactericidal for these isolates, so MBEC of *Q. brantii* extracts in combination with NAC was not performed for these isolates.

Pseudomonas aeruginosa (ATCC 27853) was a strong-biofilm producing strain and was resistant *to Q. brantii* extracts. In contrast, UTMC17 had less ability to form biofilm. Therefore, extracts easily penetrated into the thin biofilm of this isolate and were able to destroy biofilm at the lower concentrations. Shakeri et al. (25) investigated the penetration rate of anti-microbial agents such as hydrogen peroxide in biofilms with different thicknesses. Their results suggested that in the presence of biofilm barrier, the strains react to antimicrobial compounds based on the strength and thickness of their biofilms.

Water-methanol and methanol extracts at a concentration of 1/2 MIC resulted in increased biofilm formation ability (negative inhibition percentage) of *P. aeruginosa* (ATCC 27853), UTMC6 and UTMC7, respectively. Similar to our results, Taganna et al. reported that, sub-MIC concentrations of *Terminalia catappa* L. increased biofilm formation ability of *P. aeruginosa* by 220% (26). Also, Carneiro et al. assayed sub-MIC concentrations of *Croton nepeti-folius* Bail. extracts against some bacterial strains and reported that lower concentrations of extracts stimulated *P. aeruginosa* biofilm formation (27). Bahar et al. also investigated the effects of *Q. brantii* subsp. *persica* against bacterial biofilms. According to their results, the average of biofilm formation inhibition by *Quercus brantii* subsp. *persica* at a MIC50 in *P. aeruginosa*, *Escherichia coli*, *Staphylo-coccus epidermidis* and *Staphylococcus aureus* strains were 35%, 45%, 57% and 61%, respectively (28). In another study, Chusri et al. established the effect of *Q. infectoria* extract on staphylococcal biofilms. The extract at MIC of 0.25 mg/ml was significantly reduced the biofilm formation of the isolates ($P < 0.05$) (29).

Concurrent use of extract and the compounds increasing biofilm permeability provides quick and easy penetration of extract into the biofilm and facilitates the access of plant extract to the cells accumulated in the biofilm. N-acetylcysteine is a non-antibiotic drug with bactericidal characteristics. NAC can reduce biofilm formation, prevent adhesion of the bacteria to the surface, inhibit the production of the exopolysaccharide and increase biofilm destruction as well as cause death in cells sticking to the biofilm. This effect is entirely dependent on the concentration of NAC (30). Manniello et al. (31) reported that simultaneous use of macrolide antibiotics (clarithro-mycin) and NAC is useful to remove *P. aeruginosa* biofilms in the lungs of patients with cystic fibrosis. Our results also showed that simultaneous use of NAC (at the concentration without bactericidal effect) and plant extracts improved extract activity by reducing MBEC of the extracts.

Conclusion

Our results show that *Q. brantii* extracts had a suitable inhibitory effect on biofilm formation ability of *P. aeruginosa* and could eradicate preformed-biofilms in combination with NAC. More understanding of the mechanism of action and the natural components of these extracts seems to be valuable.

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